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IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF :
BERNARD WEILL : EXAMINER: RAE, CHARLESWORTH
SERIAL NO: 10/583,135 :
FILED: DECEMBER 26, 2006 : GROUP ART UNIT: 1614
FOR: USE OF SUPEROXIDE :
DISMUTASE MIMETICS AND
REDUCTASE GLUTATHIONE IN THE
FORM OF ANTICANCER DRUGS

DECLARATION UNDER 37 C.F.R. 1.132

COMMISSIONER FOR PATENTS
ALEXANDRIA, VIRGINIA 22313

SIR:

I, _____ hereby declare:

1. I am employed by _____ as an _____ and have experience in the field

_____.

2. I am familiar with the specification of the above-identified patent application and the claims on file.

3. The following experiments were performed by me or under my direct supervision.

**I - In Vivo Effect of Mangafodipir and Other Oxidative Stress Modulators on
Hematologic Toxicity of Paclitaxel and Susceptibility of Mice to Bacterial Infection**

The effect of mangafodipir and three other oxidative stress modulators in combination with paclitaxel in BALB/c mice was investigated.

In Vivo Hematologic Toxicity Analysis

BALB/c female mice between 6 and 8 weeks of age (Iffa Credo, L'Arbresles, France) were used in all experiments. PBS (1.54 mM potassium phosphate monobasic, 155.17 mM sodium chloride, 2.71 mM sodium phosphate dibasic; Life Technologies) at pH 7.2 was used as the vehicle. Mice were injected intraperitoneally with vehicle alone; with paclitaxel (Taxol), mangafodipir, MnTBAP, CuDIPS, or N-acetylcysteine alone; or with paclitaxel and one of the oxidative stress modulators in combination. Five mice were treated in each group. Paclitaxel (20 mg/kg) or vehicle alone was administered on days 0, 2, and 4. This dose of paclitaxel was used because it was previously associated with a 50% reduction in tumor volume of CT26 at day 15 without any substantial clinical toxicity. On days 0, 2, 4, and 7, mice were administered 10 mg/kg of mangafodipir, MnTBAP, or CuDIPS, 150 mg/kg of N-acetylcysteine, or vehicle. Ten days after the first injection of paclitaxel or oxidative stress modulator, mice were killed by cervical dislocation. Spleen, bone marrow from femoral bone, and 1 mL of blood were then collected. The total number of hematopoietic cells in bone marrow and leukocytes from spleen and blood were counted using a Malassez cell. The results are shown in the attached Table 1.

After 10 days of treatment with paclitaxel alone, a statistically significant decrease in the absolute number of peripheral lymphocytes, neutrophils, and monocytes was observed in blood drawn from treated mice compared with blood drawn from control mice (attached Table 1). This decrease was accompanied by a decrease in total bone marrow cell numbers. Administering mangafodipir or N-acetylcysteine in combination with paclitaxel abrogated the hematologic toxicity of paclitaxel. Neither mangafodipir nor N-acetylcysteine seemed to enhance hematopoiesis by itself. Indeed, blood and bone marrow cell counts in mice treated with mangafodipir or N-acetylcysteine alone were similar to those in untreated mice. By

contrast to mangafodipir, the two other SOD mimics, CuDIPS and MnTBAP, did not alter the hematologic toxicity of paclitaxel.

Susceptibility of Mice to Bacterial Infection

Because bacterial infection is the main complication of neutropenia, the susceptibility of mice to *S. aureus* was tested after administration of paclitaxel alone or in combination with mangafodipir.

BALB/c female mice were injected intraperitoneally with either vehicle alone, paclitaxel (60 mg/kg) alone on days 0, 3, and 6, or paclitaxel (60 mg/kg) in combination with mangafodipir (10 mg/kg) on days 0, 3, and 6. A sublethal dose of the pathogen *Staphylococcus aureus* (200 μ L of a stock suspension with an optical density of 600 at 550 nm) was then injected intraperitoneally on day 9. The survival rate of mice was evaluated 48 hours following *S. aureus* injection. A total of 17 mice were treated in each group in three independent experiments.

The results are shown in the attached Table 2.

No deaths were observed in untreated mice inoculated with a sublethal dose of *S. aureus*. By contrast, 14 of 17 mice died within 48 hours after inoculation with the same dose of *S. aureus* following treatment with paclitaxel at a dose that induced neutropenia. However, only three of 17 mice inoculated with the bacteria died when mangafodipir had been administered in combination with paclitaxel ($P < .001$).

II- Effect of Mangafodipir and Other Oxidative Stress Modulators in Mice Implanted with CT26 Tumor Cells and Treated With Paclitaxel

Mice bearing CT26 tumors were treated with either paclitaxel, mangafodipir, MnTBAP, CuDIPS, or N-acetylcysteine alone or with paclitaxel in combination with one of these oxidative stress modulators.

Each BALB/c female mouse was injected subcutaneously in the back of the neck with 2×10^6 CT26 cells. When the tumor reached a size of 200 – 500 mm³, mice were grouped in 10 groups so that the sizes of the tumors were not statistically significantly different by group. Mice were injected intraperitoneally with vehicle alone; with paclitaxel, mangafodipir, MnTBAP, CuDIPS, or N-acetylcysteine alone; or with paclitaxel and one of these oxidative stress modulators in combination. Paclitaxel (20 mg/kg) or vehicle was administered on days 0, 2, and 4. Then 10 mg/kg mangafodipir, MnTBAP, or CuDIPS or 150 mg/kg N-acetylcysteine or vehicle were administered three times a week for 1 month. Ten mice were treated in each group. Tumor size was measured with a Vernier calliper every 3 days at the same time that the oxidative stress modulator was administered. Tumor volume (TV) was calculated as follows:

$$TV \text{ (mm}^3\text{)} = (L \times W^2) / 2$$
, where L is the longest and W the shortest radius of the tumor. Results are expressed as the mean of tumor volume within each group. Tumor volumes were compared across treatment groups after each tumor size measurement, i.e., every 3 days.

The results are shown on the attached Figure 1.

Mice treated with paclitaxel alone developed smaller tumors than untreated mice (Fig 1, A-D). MnTBAP (Fig 1, A) and CuDIPS (Fig 1, B) had only minimal antitumoral effects when administered alone and did not amplify the anti-tumoral effect of paclitaxel. Mangafodipir (Fig 1, C) inhibited tumor growth when administered alone to a similar extent

as paclitaxel, and also slightly amplified the antitumor effect of paclitaxel (Fig 1, C). By contrast, mice treated with N-acetylcysteine alone developed larger tumors than untreated mice, and adding N-acetylcysteine to paclitaxel abrogated the antitumor effect of paclitaxel (Fig. 1, D).

The undersigned declares further that all statements made herein of his/her own knowledge are true and that all statements made on information and belief are believe to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

Name

Signature

Date

Table 1. Hematologic toxicity of paclitaxel administered alone or in combination with oxidative stress modulators in mice
MnTBAP = manganese [III] tetrakis-(5, 10, 15, 20)-benzoic acid porphyrin;

CuDIPS = copper [II] diisopropylsalicylate;

NAC = N-acetylcysteine.

MnDPDP = mangafodipir

Cell numbers are expressed as mean numbers of cells/ μ L (blood), 10^3 cells/ μ L (spleen) or 10^4 cells/ μ L (bone marrow).

BALB/c female mice were injected intraperitoneally with 20 mg/kg paclitaxel (on day [D] 0, D2, D4) and/or 10 mg/kg MnDPDP, MnTBAP, or CuDIPS or 150 mg/kg N-acetylcysteine (NAC) (D0, D2, D4, D7). One group of mice remained untreated. Five mice were treated in each group.

All mice were killed on day 10 and spleen cells, bone marrow cells, and blood leukocytes were counted.

Experiment	Cell number				
	Spleen	Bone marrow	Total	Neutrophils	Lymphocytes
Controls					Monocytes
Taxol	42.2 \pm 4.4	12.3 \pm 0.8	7600 \pm 678	1544 \pm 151	5584 \pm 481
MnTBAP	44.2 \pm 2.6	6.7 \pm 0.5	3400 \pm 621	222 \pm 48	3054 \pm 563
Taxol/MnTBAP	47.2 \pm 4.6	12.0 \pm 0.5	7800 \pm 561	1452 \pm 175	5784 \pm 371
CuDIPS	49.8 \pm 4.4	7.8 \pm 0.9	4375 \pm 214	392 \pm 51	3573 \pm 214
Taxol/CuDIPS	45.6 \pm 5.4	13.2 \pm 1.2	7600 \pm 430	1888 \pm 218	5376 \pm 359
MnDPDP	46.2 \pm 2.2	8.4 \pm 0.8	4200 \pm 561	534 \pm 136	3367 \pm 370
Taxol/MnDPDP	50.0 \pm 4.3	13.3 \pm 0.8	7250 \pm 387	1527 \pm 265	5347 \pm 371
NAC	48.4 \pm 4.4	12.6 \pm 0.8	6900 \pm 400	1244 \pm 100	5264 \pm 288
Taxol/NAC	43.6 \pm 6.9	12.2 \pm 1.2	7600 \pm 245	1564 \pm 208	5612 \pm 355
	44.8 \pm 5.7	11.3 \pm 1.0	7400 \pm 458	1264 \pm 80	5772 \pm 361
					364 \pm 73

Table 2. Survival rate of *Staphylococcus aureus*-injected mice

BALB/c female mice were injected intraperitoneally with 60 mg/kg paxitaxel (on day [D] 0, D3, D6) alone or in combination with 10 mg/kg mangafodipir (D0, D3, D6). One group of mice remained untreated. A sublethal dose of the pathogen *S. aureus* was injected intraperitoneally at D9 to all mice, and the survival rate was evaluated at 48 hours. A total of 17 mice were treated in each group in three independent experiments.

	Experiment 1	Experiment 2	Experiment 3	Total
	Survival			
Untreated	5/5	6/6	6/6	17/17
Endoxan	0/5	0/6	0/6	0/17
Taxol	0/5	2/6	1/6	3/17
Taxol/MnDPDP	2/5	6/6	6/6	14/17

Figure 1. Antitumor activity of Taxol (paclitaxel) with or without concomitant exposure to oxidative stress modulators in mice implanted with murine CT26 colon cancer cells. Mice (10 groups of 10 mice each) were injected with CT26 cells and then received either taxol (squares), an oxidative stress modulator (A, manganese [III] tetrakis-(5,10,15,20)-benzoic acid porphyrin [MnTBAP] ; B, copper [II] diisopropylsalicylate [CuDIPS] ; C, mangafodipir (MnDPDP) ; D, N-acetylcysteine [NAC]), or both. One group of mice remained untreated (controls). Paclitaxel (20 mg/kg) was administered intraperitoneally three times a week for 1 week. N-acetylcysteine (150 mg/kg) or mangafodipir, MnTBAP, or CuDIPS (10 mg/kg) was administered intraperitoneally three times a week for 1 month. Mean tumor volumes were determined three times a week by calipers; results are mean \pm SEM.

